*Journal of Chromatography*, 276 (1983) 202—207
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1719

## Note

# Morphine analysis by high-performance liquid chromatography

J.A. OWEN and D.S. SITAR

*Geriatric Clinical Pharmacology Unit, Departments of Medicine and of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba R3E 0W3 (Canada)*

(First received December 21st, 1982; revised manuscript received March 18th, 1983)

Several techniques for the analysis of the low concentrations of morphine (< 200 ng/ml) in biological samples have been reported including gas—liquid chromatography with electron-capture detection (GLC—ECD) [1, 2], high-performance liquid chromatography (HPLC) with amperometric (electrochemical) detection (HPLC—AD) [3—5], thin-layer chromatography [6, 7], and radioimmunoassay [8]. However the use of these techniques is not without difficulties. The necessity of sample volatility for GLC analysis requires that morphine be derivatized, commonly by acylation of the hydroxyl functions with a polyfluorinated anhydride. This procedure enables the quantitation of morphine by electron-capture detection, a process which is essentially halogen specific. Radioimmunoassay techniques for morphine analysis are exquisitely sensitive (50 pg/ml plasma [8]), but suffer from a potential lack of selectivity. Morphine-3-glucuronide, the major metabolite of morphine has approximately 10% the potency of morphine in displacing radiolabelled ligand from the antibody binding site [9], thus generating a potential source of error. HPLC, on the other hand, requires no derivatization step for either the chromatographic separation or detection of morphine. Although several reported methods utilize ultraviolet light absorbance (UV) detection the minimum detectable quantity of morphine by this technique is quite large [10]. Alternatively the use of HPLC—AD increases the sensitivity to morphine by 100-fold over UV detection techniques. By this method morphine is quantitated amperometrically by the electrochemical oxidation of the phenolic hydroxyl group of morphine [5].

Irregardless of the chromatographic technique employed for the analysis of morphine in biological samples, an initial separation of morphine from endogenous interfering compounds is necessary. Commonly the biological sample is extracted with a mixture of a chlorinated hydrocarbon or toluene

0378-4347/83/$03.00 

and an alcohol [1, 2]. Unfortunately these methods frequently result in emulsion formation which renders further sample processing difficult [2].

In this paper we describe a morphine extraction and HPLC—AD assay capable of detecting 1 ng morphine per ml plasma, and which obviates the necessity of sample derivatization and commonly encountered extraction problems. The results obtained for plasma samples containing morphine with this HPLC—AD method and a previously reported GLC—ECD morphine assay are compared.

## EXPERIMENTAL

### *Materials*

Reagent-grade chemicals and solvents were used throughout. The derivatizing agent, pentafluoropropionic anhydride (PEPA) was purchased from Pierce (Rockford, IL, U.S.A.). Morphine sulfate for the preparation of plasma standards and normorphine free base were obtained from Health and Welfare Canada (Ottawa, Canada). The internal standard for the GLC—ECD assay, N-ethyl normorphine, was synthesized from normorphine by the procedure of Ebbighausen et al. [11].

All glassware was siliconized with 10% Surfasil® (Pierce) in hexane followed by three rinses in 95% ethanol and oven drying (60°C, 12 h).

### *Apparatus*

GLC—ECD morphine analysis was performed using a Model 5713 gas—liquid chromatograph equipped with a pulsed $^{63}Ni$ electron-capture detector (Hewlett-Packard, Mississauga, Canada). The sample was separated on a 1.8 m × 2 mm silanized glass column packed with 3% OV-17 on Gas-Chrom Q, 100—120 mesh (Chromatographic Specialties, Brockville, Canada) at 210°C with a detector and injector temperature of 250°C. The carrier gas (5% methane in argon) flow-rate was 30 ml/min.

For the HPLC—AD separation of morphine a liquid chromatograph was assembled of the following components: M-6000 constant flow pump; 25 cm × 4 mm $C_{18}$ reversed-phase ($\mu$Bondapak) column (10 $\mu$m particle size); a WISP Model 710B automatic sample injector (Waters Assoc., Mississauga, Canada) and a Model 4A electrochemical detector with a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) operated at + 0.65 V with respect to an Ag/AgCl reference electrode. The mobile phase was a mixture of methanol—water—ammonium hydroxide (50:50:0.1) degassed by continual stirring, and delivered at a flow-rate of 1.3 ml/min. Chromatogram peak areas and heights were determined by an electronic integrator (HP3353 data system, Hewlett-Packard).

### *GLC assay*

The GLC—ECD assay for morphine was a modification of the method of Dahlstrom et al. [1]. The alterations involved the use of 5.0 ml 25% *n*-butanol in toluene as the extraction solvent instead of 3.0 ml toluene—butanol (9:1). In addition all extractions were performed twice with a second 5.0-ml aliquot of extraction solvent. All solvent evaporations were carried out in vacuo on a centrifugal evaporator (Savant Instruments, Hicksville, NY, U.S.A.).

*HPLC analysis*

To a PTFE-lined screw-capped test-tube were added plasma (0.5 ml) and acetonitrile (2.0 ml) containing the internal standard normorphine (100 ng/ml). The tube was capped, shaken (15 min) and centrifuged (250 *g*, 15 min). The morphine-containing supernatant fluid was decanted into a second test-tube containing extraction solvent (3.0 ml 10% *n*-butanol in chloroform) and 0.1 *M* hydrochloric acid (1.0 ml). The tube was capped, shaken and centrifuged as before. The aqueous phase (upper) was removed and added to a third tube containing extraction solvent (3.0 ml), 1 *M* sodium hydroxide solution (0.1 ml), ammonium chloride buffer (1 ml of 1 *M* ammonium hydroxide solution titrated to pH 9.0 with 2 *M* hydrochloric acid), capped, shaken (15 min) and centrifuged (250 *g*, 15 min). The organic phase was removed and evaporated in a 5-ml Reacti-Vial (Pierce) under a stream of nitrogen. The residue was redissolved in methanol (200 μl) and 20 μl injected onto the HPLC column.

*Statistical analyses*

Standard curves were determined by a least-squares linear regression procedure. Comparisons between data were performed by a 2-tail Student *t*-test.

## RESULTS AND DISCUSSION

Sample chromatograms for plasma containing no morphine and for plasma with 15 ng/ml morphine by the HPLC—AD method are shown in Fig. 1. For the HPLC—AD morphine assay standard curves obtained by plotting either the ratio of morphine to normorphine peak areas or peak heights against known sample concentrations of morphine were linear over the concentra-

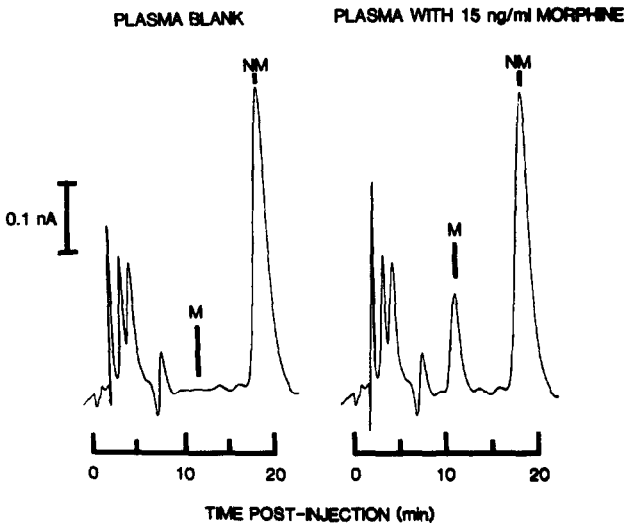


Fig. 1. Representative chromatograms for the elution of morphine (M) and normorphine (NM) by high-performance liquid chromatography with electrochemical detection. Patient samples after a drug dose yielded chromatograms essentially the same as that seen by addition of morphine to drug-free plasma.

TABLE I

COMPARISON OF GLC AND HPLC FOR THE QUANTITATIVE ESTIMATION OF MORPHINE IN PLASMA

| Method | Known concentration (ng/ml) | Observed concentration (ng/ml) | | |
|---|---|---|---|---|
| | | Mean | Standard deviation | Coefficient of variation (%) |
| GLC—ECD | 80 | 80.3 | 4.1 | 5.1 |
| | 20 | 21.0 | 2.3 | 9.1 |
| | 8.0 | 7.6 | 1.4 | 18.4 |
| | 2.0 | 2.6 | 2.0 | 76.9 |
| HPLC—AD | 100 | 100.6 | 4.1 | 4.1 |
| | 25 | 24.8 | 2.8 | 11.3 |
| | 8.0 | 8.2 | 1.4 | 17.2 |
| | 2.0 | 2.4 | 0.75 | 31.5 |

tion range examined (1.6—400 ng/ml) with intercepts not significantly different from zero. For the GLC—ECD morphine assay separate standard curves were constructed for plasma samples containing both high (160—20 ng/ml) and low (20—2 ng/ml) concentrations of morphine. In each case a plot of the ratio of morphine to N-ethyl normorphine peak areas against known standard concentrations was linear and not significantly different from zero in intercept. The between-day reproducibility over eight separate occasions of the estimation of morphine contained in four plasma samples of known concentration by each method is indicated in Table I. It is apparent from this table that both methods accurately assessed the concentration of morphine in the samples over a wide range. For the analysis of those samples containing morphine at concentrations equal to and in excess of 8 ng/ml plasma the precision of morphine quantitation, as indicated by the coefficient of variation, was similar for the two methods. However the HPLC—AD morphine assay was more reliable for assessing plasma concentrations less than 8 ng/ml even though only 0.5 ml of plasma was used as opposed to 1.0 ml by the GLC—ECD method. The coefficient of variation for the analysis of a 2 ng/ml plasma standard by HPLC—AD was 31.5% in comparison to 76.9% for the GLC—ECD technique. In this light the minimum detectable quantity of morphine, defined as a peak twice the height of the baseline noise, is in excess of 2 ng/ml plasma for the GLC—ECD assay and approximately 1 ng/ml for the HPLC—AD method.

The sample preparation for the HPLC analysis of morphine in plasma represents a departure from the procedures commonly used. Both halogenated hydrocarbon—alcohol and toluene—alcohol mixtures have been widely used for the extraction of morphine from biological samples. However, emulsions are frequently produced by shaking either of these solvent mixtures with plasma (and other protein-containing fluids) making phase separation difficult. To overcome this problem, column extraction of morphine following absorption of the aqueous phase on an inert support such as cellulose powder [12], silica [13], or gauze sponges [14] has been reported. The ex-

traction procedure described in this paper circumvents the problem of emulsion formation and the difficulties of column extraction techniques by an initial acetonitrile denaturation of plasma proteins prior to solvent extraction. Following the addition of acetonitrile to the plasma samples, the plasma proteins form a hard pellet on centrifugation, allowing the morphine-containing supernatant liquid to be decanted. Since acetonitrile is completely miscible with aqueous solutions there is no phase separation nor the associated loss of morphine following an extraction step.

For optimal extraction of morphine, an amphoteric compound, into an organic solvent, the pH of the aqueous phase must be adjusted to 8.96 [15]. An ammonium chloride buffer solution was selected over the more commonly employed carbonate buffer solutions [1, 6] due to the greater pH stability of the former on storage [2]. Although less polar solvents, such as toluene—butanol and benzene—butanol, provide cleaner extracts, the use of more polar solvents (chloroform—butanol) results in less critical pH adjustment, higher extraction efficiencies and reduced sample adsorption to glass surfaces [2]. The overall extraction efficiency of morphine for the HPLC—AD method (85%) is greater than that reported for the GLC—ECD method of Dahlstrom et al. (67%) [1], presumably due to the greater extraction recovery obtained through the use of chloroform—butanol.

In man, normorphine has been reported to be a minor metabolite not detectable in plasma and accounting for approximately 1% of the morphine dose excreted in the urine [16]. Hence endogenously produced normorphine causes little or no interference with the added normorphine internal standard (200 ng per sample). Other opiates could have been employed as an internal standard, e.g. nalorphine [4] or N-ethyl normorphine [1], provided they possess a free phenolic hydroxyl group necessary for electrochemical activity [4].

The method described for the HPLC—AD analysis of morphine presents several advantages over the GLC—ECD method described by Dahlstrom et al. [1]. It combines increased sensitivity for morphine quantitation (1 ng/ml plasma) with a simplified extraction procedure of a smaller (0.5 ml) sample and the absence of a derivatization process. The assay is sufficiently reliable and predictable that analytical runs of 50 h are routinely performed unattended with automatic sample injection and peak quantitation by electronic integrator. Prepared samples are stable at −20°C (no noticeable degradation over three months) such that samples may be processed in batches and stored for subsequent analysis. Over 1000 analyses have been performed on one reversed-phase column without significant deterioration.

ACKNOWLEDGEMENTS

The authors thank Mr. J. Sherwin for excellent technical assistance. This investigation was supported by a grant from the Canadian Foundation for the Advancement of Clinical Pharmacology and a post-doctoral Fellowship for J.A.O. by the Medical Research Council of Canada.

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